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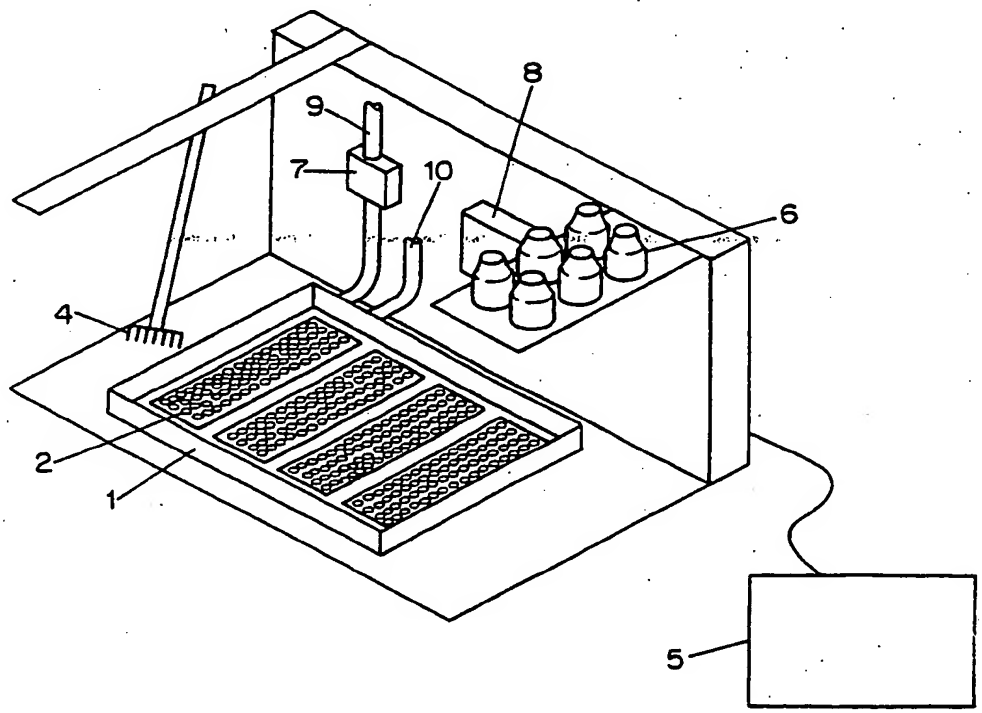
(54) Title: METHOD FOR PREPARATION OF CLOSED CIRCULAR DNA

(57) Abstract

Closed circular DNA can be recovered from a cells or subcellular organelles containing closed circular DNA by a method comprising, in sequence, the steps of: (a) lysing the cells or organelles to release DNA, for example with a combination of lysozyme and heat or detergent; (b) enzymatically treating the released plasmid DNA with a proteolytic enzyme such as Proteinase K; (c) enzymatically treating the proteolytic enzyme-treated plasmid DNA with RNase and topoisomerase (I); (d) selectively enzymatically digesting the non-circular nucleic acids from the microorganism; and (e) recovering closed circular DNA. The method is particularly applicable to recovery and purification of plasmid DNA from *E. coli*.



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Description

Method For Preparation of Closed Circular DNA

Background of the Invention

This application relates to a new method for preparing plasmid and other closed circular DNA which is readily automated.

Plasmids are small circular pieces of DNA found naturally and as a result of human intervention in many bacteria, particularly E. coli. Because many copies of an individual plasmid are frequently present within the host organism, and because plasmids are both reproduced and in many cases expressed by the host organism, plasmids have come to play a major role in many biotechnological processes. Indeed, the preparation of plasmid DNA containing a nucleic acid fragment of interest is a routine procedural precursor to such other procedures as DNA sequencing, restriction digestion, cloning, probing, amplification (e.g., PCR), hybridization, in vitro transcription or mutagenesis.

At present, there are two methods which are commonly used to prepare plasmid DNA from cultures of E. coli: the alkaline lysis method and the boiling method. Sambrook et al., Molecular Cloning, 2d ed., p. 1.21 (1989). Both methods depend on the use of centrifugation to purify plasmid at several stages of the procedures, including centrifugation of chromosomal DNA, centrifugation of phenol and chloroform extractions, and centrifugation of the ethanol precipitate. In the alkaline lysis method, sodium hydroxide and sodium dodecyl sulfate (SDS) are used to lyse cells, after which acetate and acetic acid are added to precipitate chromosomal DNA. The precipitated DNA is removed by centrifugation and the supernatant is recovered and extracted with 1:1 phenol:chloroform.

Plasmid DNA is then precipitated by adding ethanol and recovered by centrifugation. Finally, RNA is removed from the recovered plasmid DNA by resuspending the material in buffer containing pancreatic RNase.

The boiling method for preparing plasmids uses lysozyme in a boiling water bath to lyse the cells. Chromosomal DNA and cellular debris are removed by centrifugation and the supernatant containing the plasmid DNA is treated with sodium acetate and isopropanol to precipitate the plasmids. This precipitate is recovered by centrifugation, and treated with RNase.

One commercially available instrument for preparing plasmid DNA contains a built in centrifuge and isolates plasmid DNA using the alkaline lysis method. The throughput of this machine is limited to about 40 plasmid preps per day, the equivalent of what a single technician can achieve by hand in one day. The human genome project and other efforts which could involve the need for hundreds of thousands of plasmid preparations before completion make it clear that this throughput level is unacceptable. There exists, therefore, a real need for a preparation technique which can be performed rapidly and simply by automated equipment. This invention satisfies that need.

Summary of the Invention

In accordance with an embodiment of the invention, closed circular DNA can be recovered from cells or subcellular organelles containing closed circular DNA by a method comprising, in sequence, the steps of:

(a) lysing the cells or organelles to release DNA, for example with a combination of lysozyme and heat or detergent;

(b) enzymatically treating the released plasmid DNA with a proteolytic enzyme such as Proteinase K;

(c) enzymatically treating the proteolytic enzyme-treated plasmid DNA with RNase and topoisomerase I;

(d) selectively enzymatically digesting the non-circular nucleic acids from the microorganism; and

(e) recovering closed circular DNA.

The method is particularly applicable to recovery and purification of plasmid DNA from E. coli.

Brief Description of the Drawings

Fig. 1 shows an automated instrument for performing the method of the invention;

Fig. 2 shows a modified microtiter sample holder useful in automating the present invention.

Figs. 3 and 4 show cross sections of a single sample well.

Detailed Description of the Invention

The present invention is applicable generally to the isolation of closed circular DNA, both single and double stranded, from other DNA. Thus, the invention is applicable both to the preparation of plasmid samples and to preparation of circular phage DNA (e.g. phage M13 which is frequently used as a cloning vector) and DNA isolated from organelles such as chloroplasts and mitochondria. The method can also be employed in modified form to isolate viral DNA from host nucleic acids.

The first step of the method for preparing purified circular DNA in accordance with the invention is lysis of the host microorganism. For E. coli, lysis can be accomplished by a combination of enzymatic treatment with lysozyme followed by heat treatment at a

time and temperature sufficient to accomplish the result of making DNA available for subsequent enzymatic attack. Other methods for cell lysis can also be employed provided that the lysis method does not nick the plasmid DNA. For example, exposure to alkaline conditions (pH 13-14) after lysozyme treatment or species specific-lytic agents such as lysostaphin for staphylococci may be employed. Lyticase or zymolase can be used to lyse yeast cells, while detergent alone should be sufficient in the case of mammalian cells, insect cells (e.g. for baculovirus isolation), chloroplasts or mitochondria. Cellulase may be a suitable lytic enzyme for isolation from plant cells.

Following cell lysis, the cell preparation is enzymatically treated with a protease enzyme to destroy the cell structure and free nucleic acids from proteins (e.g., histones) which might interfere with subsequent enzymatic degradation steps. A suitable enzyme for this purpose is Proteinase K, a commercially available protease secreted extracellularly by Tritirachium album (Sigma Chemical Co., St. Louis, MO). Proteinase K is a serine endoproteinase of the bacterial subtilisin family that rapidly and non-specifically hydrolyzes native proteins. Conveniently, proteinase K remains active in the presence of urea and SDS and other detergents such that denaturants may be added to facilitate the proteolytic digestion and is active at relatively high temperatures which allows accelerated proteolysis. Detergents, such as TRITON X-100, may also be added to facilitate the proteolytic digestion.

After incubating the lysed bacteria in the protease enzyme for a period of time sufficient to substantially clarify the preparation, the protease enzyme is inactivated to prevent degradation of subsequently added enzymes. This inactivation is conveniently done by heating the preparation, which not

only inactivates the protease but also denatures substantially all of the bacterial DNA other than supercoiled and relaxed closed plasmid DNA into single stranded form. Other inactivators may be used (e.g., metal chelators, phenylmethylsulfonyl fluoride, iodoacetate or a change in pH (increasing or decreasing from optimum as appropriate for the enzyme used) or buffer conditions), however if heat inactivation is ineffective for a given protease, provided that these inactivators do not interfere with subsequent enzymatic steps (e.g., nuclease activity) or can be removed. In addition, chemical means for denaturing non-circular DNA may be employed, including the enzyme helicase, exposure to alkali (pH 13-14), acid (pH 1-4), urea, dimethylsulfoxide or dimethylformamide or other denaturing conditions.

Upon cooling the solution, the chromosomal DNA is likely to remain in single stranded form as a result of the low incidence of highly repetitive sequences in bacterial, e.g. E. coli, genomes. Thus, the chromosomal DNA, along with the RNA, is susceptible to enzymatic digestion by a variety of nucleases, including single stranded exo- and endonucleases and double stranded exonucleases. Suitable nuclease enzymes for this purpose include Mung Bean Nuclease, S1 Nuclease (US Biochemical Corp., "USB"), P1 Nuclease (Bethesda Research Labs, "BRL"), T7 exonuclease (USB), Bal 31 Nuclease (USB), Exonuclease I (USB), Exonuclease III (USB), Exonuclease VII (BRL) and Lambda Exonuclease (BRL). RNA from the microorganism is digested either before or concurrently with the DNA, depending on the conditions needed for enzymatic activity, using an RNase enzyme such as pancreatic ribonuclease (USB) or Ribonuclease T_1 (Pharmacia).

In selecting a nuclease for use in the present method, it is important to remember that some nuclease

enzymes, notably S1 nuclease and Bal 31 nuclease digest supercoiled plasmid DNA as well as single stranded DNA. For example, Bal 31 nuclease degrades single stranded DNA endonucleolytically and it hydrolyzes both strands at the end of double stranded DNA. Bal 31 nuclease will completely hydrolyze supercoiled plasmid DNA due to the fact that supercoiled plasmid exists transiently in single stranded form, which is a substrate for Bal 31. Legerski et al., J. Biol. Chem 252, 8740 (1977). To avoid unwanted digestion of plasmid DNA, enzymes of this type should be used after treatment with an enzyme such as topoisomerase I (BRL) which relaxes supercoiled plasmid DNA. Other enzymes which would achieve this same result would be a combination of DNase I (nickase), DNA ligase and ATP/NAD; or T4 DNA ligase and AMP. Both of these combinations would nick supercoiled DNA to yield a nicked, relaxed plasmid, and then repair the nick via the ligase. Relaxation of supercoiled DNA can also be achieved non-enzymatically with intercalating agents such as ethidium bromide.

On the other hand, T7 exonuclease degrades the 5′ end strand of double stranded DNA attacking only chromosomal DNA which exists as linear double strands. Ausubel et al., eds, Current Protocols in Molecular Biology, p. 3.12.1. T7 exonuclease will not degrade either supercoiled or relaxed plasmid. In fact, the resistance of plasmids to degradation serves as a quality control test for T7 exonuclease purity used by the manufacturer (USB).

Other options for selective removal of chromosomal DNA include the use of DNA polymerase enzymes, which under certain conditions (absence of dNTP′s and excess pyrophosphate, i.e., about 100mM) catalyze the reverse polymerization of double stranded nucleic acids. This activity requires a free 3′-OH group and thus is specific for linear (non-plasmid) DNA. These enzymes

also have exonuclease activity for either double or single stranded DNA. Kornberg, DNA Replication, W.H. Freeman & Co., pp 127-130 (1980). Suitable enzymes include Klenow fragment, T7 DNA polymerase (USB), Taq polymerase and T4 DNA polymerase.

Additional enzymes might be used in conjunction with a reverse polymerization system to enhance the rate of degradation. These enzymes include T4 polynucleotidekinase to remove the 3'-terminal phosphate and to yield a 3'-OH group, enzymes selected to degrade the dNTP product, such as hexokinase and nucleoside diphosphate kinase together with appropriate reagents (ADP and glucose).

Terminal deoxynucleotidyl transferase (TdT) could also be theoretically used to achieve selective degradation of single stranded chromosomal DNA in the presence of pyrophosphate. Like DNA polymerases, this enzyme requires a free 3'-OH group, but it acts on single stranded as opposed to double stranded nucleic acids. The dNTP's released could be further hydrolyzed with the aid of the same enzyme systems discussed above. This system is not a preferred means for selective single stranded DNA removal, however, as the reaction rate is very slow.

Restriction enzymes are potentially useful in the present invention provided the plasmid to be isolated does not have the restriction site for which the enzyme is specific. A simple test can be used to assess the presence or absence of a given restriction site in a plasmid by exposing the plasmid preparation to the restriction enzyme prior to heat treatment and digestion of chromosomal DNA and running a gel to see if plasmid DNA remains. If plasmid DNA is observed, the restriction site is not present in the plasmid. If restriction enzymes are used it is appropriate to maintain chromosomal DNA in a double stranded state

until restriction digestion has occurred. This may require lowering the temperature used to denature the protease.

If the product desired is single stranded circular DNA, nucleases with single-strand endonuclease activity cannot be used as these enzymes will degrade the desired product. Thus, in this case, a combination of nucleases is selected from among single-strand exonucleases and double-strand exonucleases. Double-strand endonucleases can also be employed provided that the circular DNA does not self anneal to produce double stranded regions or if conditions (temperature/buffer) are maintained to prevent formation of double stranded regions.

The final step in the nucleic acid preparation of the present invention is the recovery of plasmid DNA from the solution. This can be accomplished by ultrafiltration, precipitation, binding to a particle or surface which binds nucleic acids such as hydroxyapatite, glass milk or DEAE beads, centrifugation or isobutanol extraction. A preferred method is the use of an ultrafiltration device which works by permeation through a molecular weight selective membrane filter.

The method of the invention will now be illustrated by way of the following, non-limiting examples.

Example 1

A 1.5 ml E. coli culture containing the cloning vector pUC118 was grown overnight to saturation. The E. coli were pelleted and resuspended in 300 µl of 100 mM Tris-Cl (tris(hydroxymethyl)aminomethane hydrochloride), 2.5 mM $MgCl_2$, 0.5% Triton X-100, pH 8.0. Lysozyme solution, 30 µl at 10 µg/µl, was added and incubated at 37°C for 30 minutes. The lysozyme alone did not create microscopically visible ruptures in the

E. coli cells. The E. coli were heated at 95 °C for 5 minutes, and then cooled to 40°C.

Proteinase K solution, 10 µl at 10 µg/µl, was added and the E. coli were incubated at 50°C for 30 minutes. This enzymatic step resulted in the complete clearing of the solution. Microscopic examination confirmed that no E. coli or fragments were present. Apparently, the lysozyme and heating step had introduced holes in the bacteria allowing the protease to enter and dissolve the cells completely. This lysed solution was again heated at 95°C for 5 minutes, then cooled to 25°C, to inactivate the proteinase K.

The contents of the tube were precipitated by adding 35 µl of 3.0 M NaOAc (pH 5.2) + 800 µl ethanol, pelleted at 12,000g for 5 minutes, and resuspended in 50 µl of TE buffer with ribonuclease (10 mM Tris-Cl, 1 mM EDTA, 20 µg/ml pancreatic ribonuclease, pH 8.0). The preparation at this point was analyzed by gel electrophoresis (0.6% agarose in 40mM TrisOAc, 2 mM EDTA, pH 8.3). Following ethidium bromide staining, evaluation of the gel indicated that the lysate consisted largely of fragmented chromosomal DNA, fragmented RNA, and intact plasmid DNA.

To the ribonuclease treated lysate were added 5 µl $MgCl_2$ (100 mM) + 10 µl KCl (200 mM) + 0.8 µl DTT (100 mM) + 10 units Topoisomerase I (USB) + 80 units T7 Exonuclease (USB). This mixture was incubated for 2 hours at 37°C. Agarose gel electrophoresis of the mixture following incubation showed some degradation of the chromosomal DNA. The RNA smear at the bottom of the gel had been removed. Apparently, T7 exonuclease has RNase activity.

To further improve DNA digestion, 4 µl $CaCl_2$ (100 mM) + 8 µl NaCl (3.0 M) + 2.5 units Bal 31 nuclease (USB) were added to the lysate. This mixture was incubated at 30°C for 2 hours. A further improvement

in the purification of the plasmid DNA is observed upon gel electrophoresis of the product, but some additional purification was deemed appropriate.

The remaining chromosomal DNA mandates additional steps to purify the plasmid DNA. To the solution was added 1 μl dithiothreitol (DTT) + 8 units Exonuclease III (USB), an enzyme that specifically degrades the ends of double stranded DNA but not plasmid DNA. This mixture was incubated at 37°C for 30 minutes or 60 minutes and the products were again evaluated by gel electrophoresis. In each case, a much cleaner preparation of plasmid DNA was obtained. In fact, the two migrating forms of pUC118 plasmid DNA (3.2 kb) could be readily visualized: supercoiled plasmid migrates fast at 2 kb (escaped topoisomerase I conversion) and relaxed, circular (or nicked) plasmid migrates at 3.2 kb. Some remaining DNA was observed, which is probably single stranded DNA, which is resistant to Exonuclease III due to the enzyme's requirement for a double stranded substrate.

At this stage in the experiment, the DNA was precipitated from the sample using 3.0 M NaOAc and ethanol and stored overnight at -20°C. The DNA was collected by centrifugation at 15,000 g for 5 minutes, washed with 0.5 ml 70% ethanol and dissolved in 40 μl Exonuclease I buffer (67 mM glycine, 6.7 mM $MgCl_2$, 10 mM mercaptoethanol, pH 9.5). Exonuclease I (USB), 25 units, was added and incubated at 37°C for 10 min, 30 min, and 2 hours. Exonuclease I incubation resulted in only a slight improvement in the purity of plasmid DNA, although more improvement might be achieved if more enzyme were used. No degradation of either the supercoiled or relaxed plasmid DNA is observed (compared to pre-exonuclease I control) due to the specificity of Exonuclease I for the ends of single-stranded DNA.

The solution was next heated at 95°C for 5 minutes and cooled to 25°C. The DNA was precipitated using 3.0 M NaOAc and ethanol and redissolved in 21 μl of 30 mM NaOAc, 200 mM NaCl, 1 mM $ZnCl_2$, pH 4.6. 236 units of S1 nuclease (USB) were then added and the mixture was incubated at 37°C for 1 min, 10 min, 30 min, and 2 hours. The result was a dramatic purification of the plasmid DNA, although some loss of even the plasmid DNA was observed if incubation times of 30 minutes or longer were used. The only band on the gel remaining was relaxed, circular plasmid DNA. This conclusion is supported by the following reasoning: Prior to S1 incubation, the sample was heat denatured at 95°. If the plasmid was either nicked or linearized, the heat would have converted it to single stranded DNA, which would be degraded by S1 nuclease.

Example 2

E. coli containing the plasmid pUC118 was grown by inoculating 500 μl of TYGPN both (2.0 g tryptone, 1.0 g yeast extract, 800 μl glycerol 0.5 g sodium phosphate and 1.0 g potassium nitrate in 100 ml of deionized water, pH 7.0.) containing 50 μg/ml ampicillin. The E. coli was incubated in a sealed 0.5 ml Eppendorf tube at 37°C without agitation or aeration for 10 hours. The cell density before harvesting was about 1.6 X 10^9 cells/ml. The cells were harvested by centrifugation at 12,000g (1 min, 4°C) and resuspended in 300 μl of 100 mM Tris-Cl, 2:5 mM $MgCl_2$, 0.5% TRITON X-100, pH 8.0.

30 μl of lysozyme (10 mg/ml) was added to the resuspended cells and the mixture was incubated at 37°C for 30 minutes. The mixture was then heated to 95°C for 5 minutes and then cooled to 50°C.

7.5 μl $CaCl_2$ solution (100 mM) and 10 μl proteinase K solution (10mg/ml) was added to the cooled mixture and incubated for 60 minutes at 50°C. The mixture was

then heated to 95°C again for 5 minutes and cooled to 25°.

Total DNA was precipitated by addition of 3.0 M NaOAc (pH 5.2) +800 µl ethanol and incubating at -20°C for 30 minutes. The precipitated DNA was recovered by centrifugation (12,000g) for 5 minutes at 4°C. The pellet was recovered, washed with 70% ethanol and dried at room temperature.

The pellet was then redissolved in 50 µl of 50 mM Tris-Cl, 50 mM KCl, 10 mM $MgCl_2$, 0.1 mM EDTA, 1.0 mM DTT, 20 µg/ml RNase (USB), pH 7.5. 30 units of Topoisomerase I (BRL) were then added and the mixture was incubated at 37°C for six hours to degrade RNA and relax supercoiled plasmid DNA. At the end of the incubation time, the mixture was heated to 95°C for 5 minutes to inactivate the enzymes, and then cooled to 25°C.

Total DNA was again recovered by precipitation using 4.5 µl of 3.0 M NaOAc (pH 5.2) and 110 µl of ethanol. After 30 minutes of incubation time at -20°C, the precipitated DNA was collected by centrifugation (12000 g, 5 min. 4°C), washed with 70 % ethanol and dried at room temperature. The resulting material, containing both plasmid and chromosomal DNA was dissolved in 40 µl of 30 mM NaOAc, 200 mM NaCl, 1 mM $ZnCl_2$, pH 4.6. 118 units of S1 nuclease (USB) were added and the mixture was incubated at 37°C for 60 minutes. The resulting solution was analyzed by agarose gel electrophoresis in 40 mM TrisOAc, 1 mM EDTA, pH 8.3 buffer. The gels showed plasmid DNA of approximately 95% purity.

In the foregoing experiment, precipitation/centrifugation was used to recover DNA at the end of two intermediate enzymatic digestion steps to allow for buffer exchange. It will be understood, however, that the use of this particular technique is not essential

to practicing the invention. Thus, ultrafiltration, performed intermittently or continuously, could be used in place of the precipitation/centrifugation steps, thus yielding a method free of centrifugation steps after the initial harvesting of the cells.

Example 3

The experiment of example 2 was repeated except that T4 DNA ligase and AMP was used in place of topoisomerase to relax the plasmid DNA. The DNA pellet was dissolved in 50 µl of 50mM Tris-Cl, 10 mM $MgCl_2$, 1mM DTE (dithioerythritol), 20 µg/ml RNase (USB), pH 7.6. To this 5 units of T4 DNA ligase (BRL) and 2.5 µl of 50 mM AMP were added. The mixture was incubated at 37°C for 6 hours and then heated to 95°C for 5 minutes before cooling to 25°C. Comparable purification to Example 2 was achieved.

Example 4

M13 DNA was prepared from a culture of E. coli transformed with M13mp19 replicative form DNA that had been grown in LB broth overnight with agitation. The culture had achieved saturation density.

400 µl of the culture were placed in an 0.5 ml Eppendorf tube. 30 µl of lysozyme solution (10 mg/ml) was added, and the mixture was incubated at 37°C for 30 minutes. After the incubation, the mixture was heated to 95°C for 5 minutes and then cooled to 50°C.

10 µl of Proteinase K solution (10 mg/ml) and 10 µl of 100 mM $CaCl_2$ were then added and the mixture was incubated at 50°C for 60 minutes, after which it was heated to 95°C for 5 minutes and cooled to 25°C. The nucleic acids in the sample were precipitated by adding 50 µl of 3.0 M NaOac (pH 5.2) and 1 ml of ethanol and incubating at -20°C for 45 minutes. The precipitated nucleic acids were collected by centrifugation (12000g, 5 minutes, 4°C), washed with 70% ethanol and dried at room temperature.

The dried nucleic acids were dissolved in 75 μl of 50 mM Tris-Cl (pH 8.0), 10 mM $MgCl_2$, 50 mM NaCl. 20 units of Mbo I (BRL), a restriction enzyme, were added. Following incubation at 37°C for 60 minutes, the mixture was heated to 95°C for 5 minutes and then cooled to 37°C. 20 units of T4 polynucleotide kinase (BRL), 2.5 μg RNase and 5 μl of 100 mM DTT were then added and the mixture was incubated at 37°C for 60 minutes.

As evaluated by agarose gel electrophoresis, this procedure resulted in a significant purification of the single-stranded M13 DNA migrating at about 5000 bp on the gels. Two other bands were visible on the gel, migrating at about 4000 and 6000 bp, which are either alternate sized intracellular forms of the closed circular single-stranded DNA or the double stranded M13 supercoiled and relaxed plasmid DNA. A small amount of single-stranded chromosomal DNA was also observed. This can probably be removed by the addition of a single-stranded exonuclease, such as DNA polymerase, to the enzyme mixture.

Example 5

A simple modification of the protocols discussed above makes it possible to use the present invention to isolate RNA from a sample. In this case, RNases are omitted and a mixture of DNases is used to degrade all DNA. Selective membrane filtration of the sample using a 25,000 dalton cut-off membrane isolates total RNA including messenger RNA, transfer RNA and ribosomal RNA.

Example 6

The method of the invention can be used to isolate viral particles. Lytic viruses produce viral particles within a host cell which are protected by viral coat proteins. Host DNA, on the other hand is exposed to the surrounding environment upon lysis of the cell.

Viral DNA/RNA can therefore be recovered, separate from host DNA, by the steps of enzymatic lysis of the cell walls or membranes, addition of nucleases to the lysed cells to degrade host nucleic acids, addition of nuclease inhibitors, proteases or heat to inactivate the nucleases and then treatment with a proteolytic enzyme to destroy the nucleases and remove the viral coat protein.

The method of the present invention lends itself readily to automation because it can be performed without resort to centrifugation, phenol extraction, ethanol precipitation or gel electrophoresis. Fig. 1 shows a schematic of a basic instrument for accomplishing this purpose.

As shown in Fig. 1, sample containers 2 are held in a temperature control block 1 and supplied with reagents via an automated pipettor 4 controlled by a computer 5. The temperature of the block can be controlled by introducing a circulating liquid (e.g. water) via line 9 to flow through the block and exit via line 10. Liquid flow can be controlled via value 7 whose operation is controlled to maintain the sample in the holders 2 at the necessary temperatures. Feedstock supplies 6 may conveniently be included within the apparatus housing 3, as may a pump 8 to provide PEG or vacuum to the sample holder as discussed further below. The sample containers may advantageously be microtiter plates having a plurality of wells, the bottom of each of which is in contact through a filtration membrane with a circulating solution of polyethylene glycol (PEG) or a vacuum. (See Fig. 2) The osmotic pressure created by the PEG or the pressure differential caused by the vacuum draws molecules of molecular weight below the cut-off of the membrane out of the sample solution, thus allowing continuous removal of low molecular weight products of proteolytic or nucleolytic digestion

and facilitating buffer exchanges when needed throughout the process.

Fig. 2 shows schematically a microtiter plate 21 adapted for use in the present invention. Each well 22 of the plate 21 is in contact through an ultrafiltration membrane 24 with a stream of PEG flowing through tube 23. PEG is supplied from vessel 25 into tube 23 and flows through the microtiter plate 21 propelled by pump 8, for example a peristaltic pump.

Fig. 3 shows a cross section of a sample well in the microtiter plate 21 and the adjoining tube 23. PEG solution 31 circulating within tube 23 past ultrafiltration membrane 24 osmotically draws water and materials of molecular weight below the filter's cut-off out of the sample well 22. The height of the impermeable wall 34 defines the final volume of the sample if buffer is not continuously added.

Fig. 4 shows an alternative sample well arrangement in which vacuum rather than osmotic pressure is employed. In this case, each sample well 22 has an ultrafiltration membrane 24 at the bottom of the well. Vacuum is supplied as needed to the bottom side of the membrane to draw water and low molecular weight solutes out of the well while leaving high molecular weight closed circular DNA behind.

Claims

1. A method for purifying circular DNA from cells or subcellular organelles of a host organism containing circular DNA comprising the steps of:

(a) lysing the cells or organelles to release circular DNA;

(b) enzymatically treating the preparation containing released circular DNA with a protease enzyme;

(c) heating the protease-treated preparation to denature substantially all non-circular DNA to single stranded form;

(d) treating the heat treated preparation with nuclease enzymes effective to selectively digest RNA and chromosomal DNA while leaving closed circular DNA intact; and

(e) recovering closed circular DNA.

2. A method according to claim 1, wherein the host organism is E. coli and the protease enzyme is Proteinase K.

3. A method according to claim 1, wherein the circular DNA is single stranded and wherein the nuclease enzymes are selected from among nucleases substantially free from single-stranded endonuclease activity.

4. A method according to claim 1, wherein the circular DNA is double stranded, further comprising the step of treating the preparation to relax supercoiled plasmid DNA prior to treatment with the nuclease enzymes.

5. A method according to claim 4, wherein the preparation is treated with topoisomerase to relax supercoiled DNA.

6. A method according to claim 4, wherein the nuclease enzymes are selected from the group consisting of Mung Bean Nuclease, S1 Nuclease, P1 Nuclease, T7 exonuclease, Bal 31 Nuclease, Exonuclease I, Exonuclease III, Exonuclease VII and Lambda Exonuclease.

7. A method according to claim 1, wherein the nuclease enzymes are selected from the group consisting of Mung Bean Nuclease, S1 Nuclease, P1 Nuclease, T7 exonuclease, Bal 31 Nuclease, Exonucleases I, Exonuclease III, Exonuclease VII and Lambda Exonuclease.

8. A method according to claim 7, wherein the host organism is E. coli and the protease enzyme is Proteinase K.

9. A method of purifying viral particles from infected host cells comprising the steps of

(a) lysing the host cells to release viral particles and host nucleic acids from the cells;

(b) adding nucleases to the lysed cells to degrade host nucleic acids;

(c) adding nuclease inhibitors or inactivators to the nuclease-treated lysed cells;

(d) treating the preparation with a proteolytic enzyme to destroy the nucleases and remove coat protein from the viral particles; and

(e) recovery to uncoated viral particles.

10. A method for separating and recovering closed circular DNA from a composition containing closed circular DNA and linear DNA comprising the steps of

(a) treating the composition to denature substantially all of the linear DNA to single-stranded form;

(b) treating the denatured composition with nuclease enzymes effective to selectively digest linear DNA while leaving closed circular DNA intact; and

(c) recovering the closed circular DNA.

11. A method according to claim 10, wherein the circular DNA is single-stranded and wherein the nuclease enzymes are selected from among nucleases substantially free from single-stranded endonuclease activity.

12. A method according to claim 10, wherein the circular DNA is double-stranded, further comprising the step of treating the composition to relax supercoiled plasmid DNA prior to treatment with the nuclease enzymes.

13. A method according to claim 12, wherein the composition is treated with topoisomerase to relax supercoiled DNA.

14. A method according to claim 12, wherein the nuclease enzymes are selected from the group consisting of Mung Bean Nuclease, S1 Nuclease, P1 Nuclease, T7 exonuclease, Bal 31 Nuclease, Exonuclease I, Exonuclease III, Exonuclease VII and Lambda Exonuclease.

15. A method according to claim, 10, wherein the nuclease enzymes are selected from the group consisting of Mung Bean Nuclease, S1 Nuclease, P1 Nuclease, T7 exonuclease, Bal 31 Nuclease, Exonuclease I, Exonuclease III, Exonuclease VII and Lambda Exonuclease.

16. A method according to claim 10, further comprising the step of treating the composition with a restriction endonuclease prior to the denaturation step, wherein the restriction endonuclease is selected such that the circular DNA to be separated and recovered is not cleaved by the restriction endonuclease.

17. An apparatus for separation and recovery of closed circular DNA, comprising

(a) a sample holder having a plurality of sample wells, each of said wells having an ultrafiltration membrane disposed in a lower portion thereof through which liquid can be removed from the well;

(b) means for controlling the temperature of the sample holder and of material within the wells;

(c) means for drawing liquid through the ultrafiltration membranes at the bottom of each well; and

(d) means for adding reagents to each of said sample wells.

18. An apparatus according to claim 17, wherein the means for drawing liquid through the ultrafiltration membranes is a vacuum pump.

19. An apparatus according to claim 17, wherein the means for drawing liquid through the ultrafiltration membranes is a manifold containing a circulating fluid which causes water to pass through the ultrafiltration membrane as a result of osmotic pressure.

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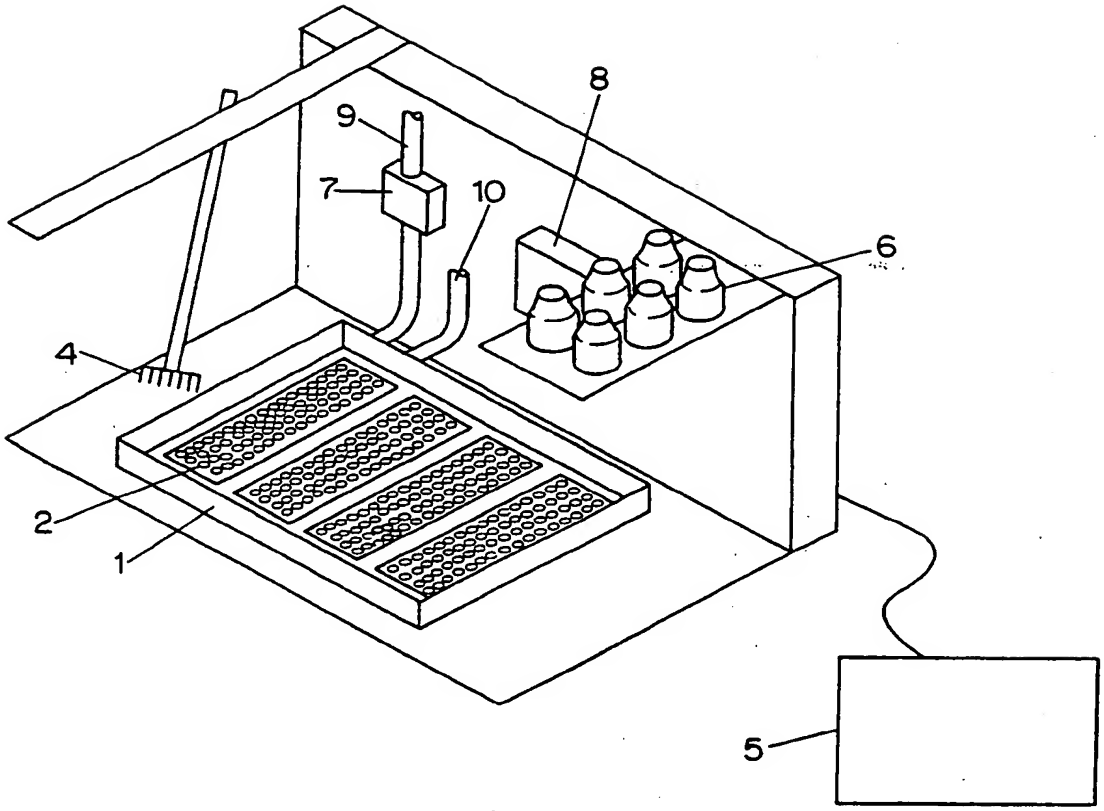

FIG. 1

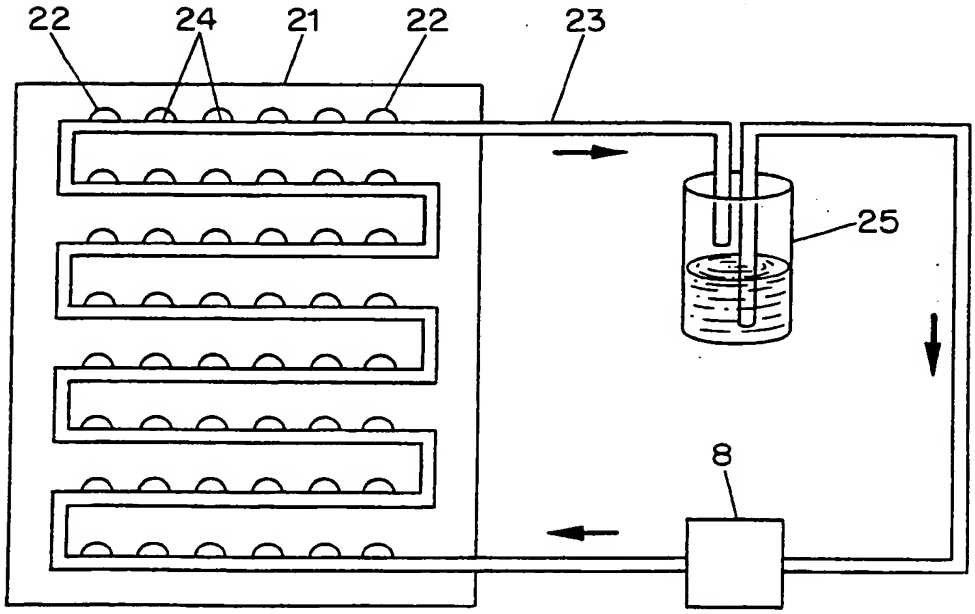

FIG. 2

SUBSTITUTE SHEET

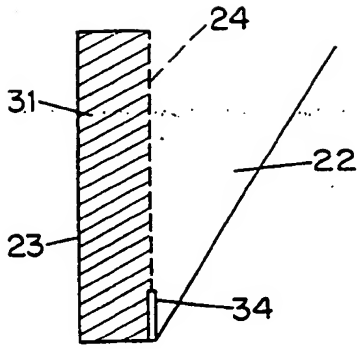

FIG. 3

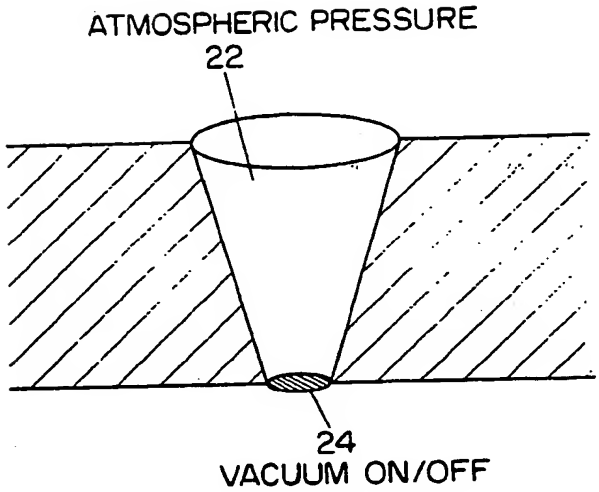

FIG. 4

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US92/00540

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[3]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC (5): C12P 19/34; C12N 7/02; C12M 1/12
US CL : 435/91, 239, 311

II. FIELDS SEARCHED

Minimum Documentation Searched[4]

Classification System	Classification Symbols
U.S.	435/91, 239, 311

Documentation Searched other than Minimum Documentation to the extent that such Documents are included in the Fields Searched[5]

APS, Biosis, World Patent Index

III. DOCUMENTS CONSIDERED TO BE RELEVANT[14]

Category*	Citation of Document,[16] with indication, where appropriate, of the relevant passages[17]	Relevant to Claim No.[18]
y	Maniatis et al., "Molecular Cloning: A Laboratory Manual", published 1982 by Cold Spring Harbor Laboratory (Cold Spring Harbor, N.Y.), see pages 80-87 and 89-91.	1-16
y	Ausubel et al., "Current Protocols in Molecular Biology", published 1989 by John Wiley (New York), see pages 3.11.1-3.11.4 and 3.12.1-3.12.3.	1-16
y	US, A, 5,047,215 (Manns), 10 September 1991, see entire document	17-19
y	Atkins, "Physical Chemistry", published 1978 by W. H. Freeman and Company (San Fransisco), see pages 222-225.	17-19

* Special categories of cited documents:[15]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search[2]	Date of Mailing of this International Search Report[2]
27 APRIL 1992	06 MAY 1992
International Searching Authority[1]	**Signature of Authorized Officer[20]**
ISA/US	Deborah Frelse for Philip W. Carter

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